

Inhibition of $\text{Na}^+\text{-H}^+$ Exchange Prevents Hypertrophy, Fibrosis, and Heart Failure in β_1 -Adrenergic Receptor Transgenic Mice

Stefan Engelhardt, Lutz Hein, Ursula Keller, Kerstin Klämbt, Martin J. Lohse

Abstract—Chronic stimulation of the β_1 -adrenergic receptor leads to hypertrophy and heart failure in β_1 -adrenergic receptor transgenic mice and contributes to disease progression in heart failure patients. The cellular mechanisms underlying these detrimental effects are largely unknown. In this study, we have identified the cardiac $\text{Na}^+\text{-H}^+$ exchanger (NHE1) as a novel mediator of adrenergically induced heart failure. β_1 -Adrenergic receptor transgenic mice showed upregulation of both NHE1 mRNA ($+140 \pm 6\%$) and protein ($+42 \pm 19\%$). In order to test whether increased NHE1 is causally related to β_1 -adrenergic-induced hypertrophy, fibrosis, and heart failure, β_1 -adrenergic receptor transgenic (TG) and wild-type (WT) littermates were treated with a diet containing 6000 ppm of the NHE1 inhibitor cariporide or control chow for 8 months. There was significant hypertrophy of cardiac myocytes in β_1 -adrenergic receptor transgenic mice (2.3-fold increase in myocyte cross-sectional area), which was virtually absent in cariporide-fed animals. Interstitial fibrosis was prominent throughout the left ventricular wall in nontreated β_1 -adrenergic receptor transgenic mice (4.8-fold increase in collagen volume fraction); cariporide treatment completely prevented this development of fibrosis. Left ventricular catheterization showed that cariporide also prevented the loss of contractile function in β_1 -adrenergic receptor transgenic mice: whereas untreated transgenic mice showed a significant decrease in left ventricular contractility (5250 ± 570 mm Hg/s TG versus 7360 ± 540 mm Hg/s WT, $\text{dp/dt}_{\text{max}}$), this decrease was completely prevented by cariporide (8150 ± 520 mm Hg/s TG cariporide). Inhibition of NHE1 prevented the development of heart failure in β_1 -receptor transgenic mice. We conclude that the cardiac $\text{Na}^+\text{-H}^+$ exchanger 1 is essential for the detrimental cardiac effects of chronic β_1 -receptor stimulation in the heart. (*Circ Res.* 2002;90:814-819.)

Key Words: transgenic mouse ■ heart failure ■ β -adrenergic receptor ■ $\text{Na}^+\text{-H}^+$ exchanger ■ cariporide

Acute stimulation of cardiac β_1 -adrenergic receptors represents the most powerful mechanism to increase heart rate and contractility.¹ Chronic stimulation of cardiac β_1 -adrenergic receptors, however, has detrimental effects on the heart. This is evident from transgenic models with cardiac overexpression of β_1 -adrenergic receptors.^{2,3} These mice develop progressive cardiomyocyte hypertrophy followed by left ventricular fibrosis and ultimately overt heart failure. The concept that chronic overstimulation of cardiomyocyte β_1 -adrenergic receptors is harmful is supported by clinical studies on heart failure patients. These patients have chronically elevated plasma catecholamine levels,⁴ and increased catecholamine levels closely correlate with the prognosis.⁵ Blockade of this sympathetic activation by the use of β -adrenergic antagonists has been shown to decrease mortality from heart failure in several large clinical trials.⁶ However, it is largely unknown which cellular mechanisms are responsible for the detrimental effects of chronic β_1 -adrenergic stimulation (for discussion, see Steinberg et al⁷).

The cardiac $\text{Na}^+\text{-H}^+$ exchanger 1 (NHE1) has recently gained considerable interest in the context of myocardial ischemia.^{8,9} Activation of this exchanger in myocardial ischemia appears to be causally related to the calcium overload observed during ischemia,¹⁰ and several studies have demonstrated protection from ischemic injury by NHE inhibition both in animal models of myocardial ischemia (MI) and in patients undergoing coronary interventions.¹¹⁻¹³ A protective effect of cariporide has recently been demonstrated in the setting of post-MI remodeling,¹⁴ where protection could be demonstrated up to 3 months after MI.

We hypothesized that the cardiac $\text{Na}^+\text{-H}^+$ exchanger 1 (NHE1) might be involved in the detrimental effects of β -adrenergic stimulation during the progression of heart failure. We observed increased levels of NHE1 in mice with cardiac-specific overexpression of the β_1 -adrenergic receptor and determined the effect of $\text{Na}^+\text{-H}^+$ exchange inhibition on the development of heart failure in this mouse model.

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From the Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany.

Kerstin Klämbt is presently working as a PhD student at Aventis Pharma, Frankfurt.

Correspondence to Martin J. Lohse, Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Straße 9, 97078 Würzburg, Germany. E-mail lohse@toxi.uni-wuerzburg.de

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Materials and Methods

Transgenic Mice

The generation of transgenic mice overexpressing the human β_1 -adrenergic receptor under the control of the α -MHC promoter has been described previously.³ Male wild-type and transgenic littermates derived from crosses of heterozygous transgenic (line β_1 TG4) and wild-type mice were studied. The animals were fed with standard animal chow containing 6000 ppm cariporide or control chow beginning at 3 weeks of age for 5 months (histological analysis) and 8 months (functional analysis), respectively. With this diet a mean plasma concentration of 2.5 ± 0.3 μ mol/L cariporide was achieved. All animal procedures were approved by the responsible university and government authorities (protocol No. 621-2531.01-10/98).

Determination of NHE1 Expression

Total RNA was prepared according to the method of Chomczynski. After preparation of the RNA, the concentration was determined by UV-absorbance and denaturing agarose gel electrophoresis was performed. The RNA was visualized using ethidium bromide staining followed by digital image acquisition with a CCD camera. All of the samples studied were free of degradation as assessed by the comparison of the band intensities of the 28S and the 18S bands compared with faster migrating signals. The 18S-band intensities of wild-type and transgenic animals were essentially identical and were used to normalize the specific RNA levels.

RNAse protection analysis was carried out essentially as described previously.¹⁵ Briefly, a 446-nt fragment of murine NHE1 was amplified from murine heart cDNA by PCR (forward primer, 5'-CTTCCTGCTGCCACCCATCA-3'; reverse primer 5'-AGACCACGCCACAAACACC-3' and subcloned into a Bluescript vector. Transcription of the radioactively labeled antisense probe was carried out using T7 polymerase (Ambion) and hybridization was allowed to occur overnight. After treatment with RNAse A and T1 for 30 minutes, samples were precipitated and electrophoresed on 5% polyacrylamide/8 mol/L urea gels. The size of the unprotected fragment was 60 nt longer than that of the protected fragments (446 nt), thus excluding the contribution of undigested probe to the signal. NHE1 protein expression was determined by Western blotting using a monoclonal antibody directed against NHE1 (Chemicon). Briefly, after homogenization of left ventricular samples in lysis buffer (50 mmol/L Tris pH 6.7, 1 mmol/L Na_2VO_4 , 2% SDS, 1 mmol/L PMSF, 10 μ g/mL leupeptin), protein concentration was determined using the biconic acid assay (Pierce), and mercaptoethanol was added to a final concentration of 2.5%. Protein (30 μ g) was loaded on 7.5% polyacrylamide gels and blotted onto nitrocellulose membranes (Schleicher and Schuell). Incubation with the primary antibody (diluted 1:5000) was carried out overnight at 4°C. The blots were quantified using [125 I]-labeled protein A (ICN Biochemicals) followed by PhosphorImager analysis. Lysates of cells overexpressing NHE1 were used as a positive control. Western blotting with an antibody directed against calsequestrin (kindly provided by Larry Jones, Indianapolis, Ind) was used to control for equal loading of the samples. Calsequestrin expression is unaltered in β_1 -adrenergic receptor transgenic mice¹⁵ and was confirmed to be unaltered in the mice investigated for the present study.

Histological and Morphometric Analysis

Midventricular slices from left ventricles were fixed with 8% paraformaldehyde in phosphate-buffered saline. After paraffin-embedding, 5- μ m sections of hearts from 5-month-old mice were stained with hematoxylin-eosin for morphometric analysis³ and with Sirius red for the detection of fibrosis by semiautomated image analysis (Lucia G, Nikon).¹⁵

In Vivo Cardiac Catheterization

Left ventricular catheterization was carried out essentially as described.³ Briefly, the right carotid artery was cannulated with a 1.4F high fidelity micromanometer (Millar Instruments), and the catheter was advanced into the left ventricle under continuous monitoring of

the pressure waveform. The data were digitized at 2000 Hz using a MacLab system (ADInstruments).

Statistical Analyses

Data are presented as mean \pm SEM. Comparison between groups was made by use of either Student's *t* test or ANOVA followed by Bonferroni's post hoc test as appropriate.

Results

Enhanced Expression of Cardiac NHE1 in β_1 -Receptor Transgenic Mice

This study was done in mice with cardiac-specific overexpression of the β_1 -adrenergic receptor. These mice develop cardiac myocyte hypertrophy, fibrosis, and eventually heart failure over several months.³ To determine the expression of the Na^+ - H^+ exchanger in the heart, we performed RNAse protection analyses with a probe specific for murine NHE1. We found a 140% increase of NHE1 mRNA in hearts of 5-month-old β_1 -receptor transgenic mice compared with wild-type controls (Figure 1A). The increase in NHE1 expression was also seen in animals as young as 8 weeks to a similar extent (data not shown). Analysis of NHE1 expression on the protein level with a monoclonal antibody specific for NHE1 confirmed a significant upregulation of NHE1 in the hearts of β_1 -receptor transgenic mice (Figure 1B). NHE1 expression was completely normalized after treatment with cariporide (Figure 1C). Similarly, no changes in NHE1 protein levels could be detected.

NHE1 Inhibition Prevents Histological Alterations in β_1 -Receptor Transgenic Mice

In order to evaluate the significance of these results, we sought to investigate whether NHE1 might play a role in β -adrenergically induced heart failure. To this end, we treated wild-type β_1 -adrenergic receptor transgenic mice with the selective NHE1 inhibitor cariporide from the age of 3 weeks on. A hallmark of the cardiac phenotype of β_1 -receptor transgenic mice is the development of myocardial fibrosis.¹⁵ We stained midventricular sections from the left ventricle with picric acid/Sirius red to detect fibrous tissue. Interstitial fibrosis became visible at 3 months of age and was prominent throughout the left ventricle at 5 months of age (Figure 2A). In wild-type animals, no fibrosis was observed at this age. In cariporide-treated transgenic animals aged 5 months, formation of left ventricular fibrosis was completely prevented (Figure 2A). Quantitative analysis of left ventricular collagen volume fraction (CVF) confirmed complete inhibition of β -adrenergically induced left ventricular fibrosis by inhibition of the Na^+ - H^+ exchanger (Figure 2B). To determine the effect of NHE1 inhibition on cardiomyocyte hypertrophy, myocyte cross-sectional areas from the left ventricular wall were analyzed morphometrically. In the untreated β_1 -receptor transgenic animals, the cross-sectional area of left ventricular cardiomyocytes was increased more than 2-fold compared with wild-type animals (Figure 2C). On treatment with cariporide, the β -adrenergically induced cardiomyocyte hypertrophy was found to be decreased by 88%.

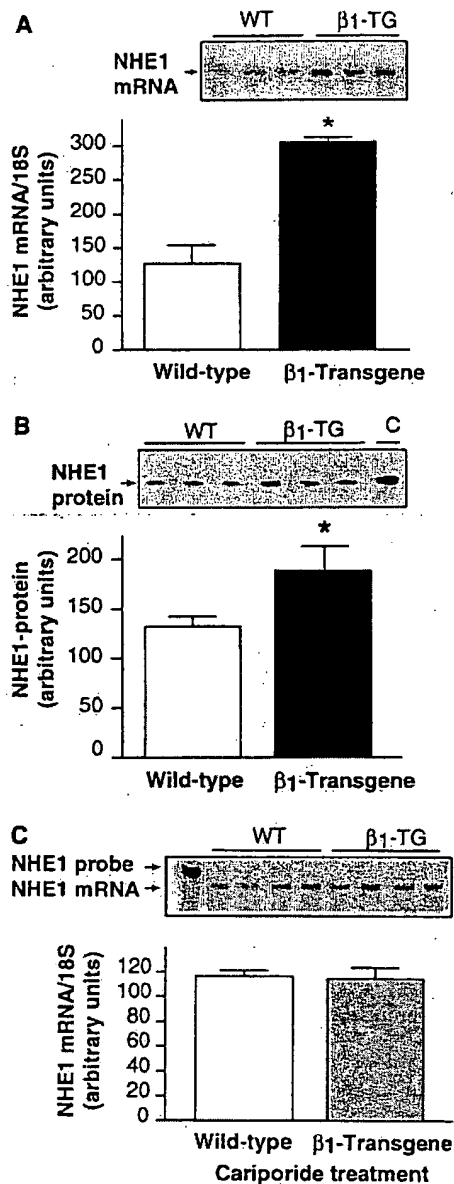


Figure 1. Enhanced expression of cardiac NHE1 in β_1 -receptor transgenic mice. **A**, NHE1 mRNA expression was determined by RNase protection analysis using a probe specific for murine NHE1. The hybridization signal specific for NHE1 expression was significantly enhanced in left ventricular RNA from β_1 -adrenergic receptor transgenic mice (5 months old) vs wild-type mice. * $P < 0.01$ TG vs WT, $n = 8$ to 9. **B**, Expression of NHE1 on the protein level was assessed by Western blotting with a monoclonal antibody specific for NHE1 (Chemicon). NHE1 protein was significantly enhanced (+42%) in left ventricular samples from β_1 -adrenergic receptor transgenic mice compared with wild-type mice. * $P < 0.05$, $n = 11$ to 12. Mice at 5 months old were used for these experiments. **C**, Expression of NHE1 after treatment with the NHE1 inhibitor cariporide. Mice were treated with cariporide for 5 months and expression of NHE1 was determined by RNase protection analysis. Treatment with cariporide abolished the increase of NHE1 seen in untreated mice.

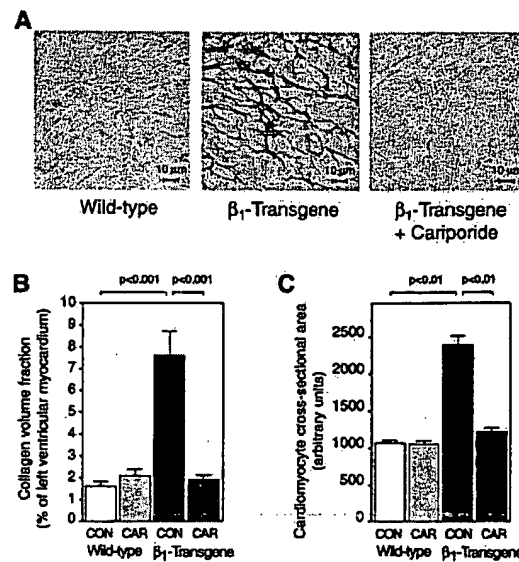


Figure 2. Prevention of β_1 -adrenergic receptor induced fibrosis by treatment with cariporide. **A**, Paraffin sections of mouse hearts were cut perpendicular to the long axis and stained with picric acid/Sirius red to assess left ventricular collagen content. At 5 months old, β_1 -transgenic animals show prominent left ventricular fibrosis throughout the left ventricle. The development of fibrosis could be inhibited completely under therapy with cariporide. **B**, Left ventricular collagen volume fraction was determined by digital image analysis of collagen staining with LuciaG software (Nikon). Data are mean \pm SEM; $n = 4$. * $P < 0.01$ TG control vs TG cariporide. **C**, Myocyte cross-sectional areas of left ventricular cardiomyocytes. Data are from 50 cardiomyocytes per group (10 cells from each animal). Treatment with the NHE inhibitor cariporide significantly reduced the development of cardiomyocyte hypertrophy.

Prevention of β_1 -Adrenergically Induced Cardiac Hypertrophy

We next determined the effect of NHE1 inhibition on physiological growth of the animals and their hearts and on β_1 -adrenergic receptor-induced cardiac hypertrophy. In untreated β_1 -adrenergic receptor transgenic animals, a significant increase in the heart weight and the heart weight/body weight ratio was observed (Figures 3B and 3C). Under treatment with cariporide, heart weight and the heart weight/body weight ratio were completely normal. Interestingly, inhibition of cardiac $\text{Na}^+\text{-H}^+$ exchange with cariporide resulted in selective inhibition of pathological hypertrophy induced by β_1 -adrenergic receptor stimulation without affecting normal growth of the animals and their hearts. Heart and body weights of wild-type animals treated with cariporide were not different from nontreated hearts (Figures 3A through 3C).

Improvement of Cardiac Function by Inhibition of the $\text{Na}^+\text{-H}^+$ Exchanger 1

Left ventricular function was determined in vivo by left ventricular catheterization of anesthetized animals. Treatment with cariporide prevented the decrease of left ventricular pressure observed in nontreated β_1 -receptor transgenic mice (Figure 4A). Cardiac contractility is increased in young β_1 -receptor transgenic animals but then progressively de-

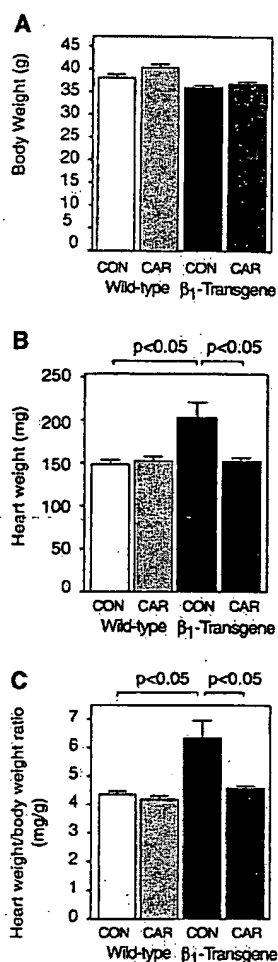


Figure 3. Inhibition of $\text{Na}^+\text{-H}^+$ exchange prevents the development of cardiac hypertrophy in β_1 -receptor transgenic mice but does not affect physiological growth. Body weight (A) and heart weight (B) of β_1 -adrenergic receptor transgenic mice and wild-type mice under therapy with cariporide. Treatment with the NHE inhibitor cariporide did not impair physiological growth of the animals but inhibited cardiac hypertrophy. C, Heart weight-to-body weight ratio is preserved in β_1 -adrenergic receptor transgenic mice treated with cariporide. Data are mean \pm SEM; $n=5$.

clines as the animals get older.³ At 8 months of age, left ventricular contractility ($\text{dp}/\text{dt}_{\text{max}}$) was significantly depressed in nontreated transgenic animals. Treatment with cariporide completely inhibited the impairment of left ventricular systolic function in β_1 -receptor transgenic mice (Figure 4B). This beneficial effect of cariporide was also observed for diastolic dysfunction in these mice (Figure 4C). Again, β_1 -receptor transgenic mice treated with cariporide did not show impairment of left ventricular relaxation compared with wild-type animals.

Inhibition of NHE1 did not affect LV systolic pressure, heart rate (481 ± 21 versus 467 ± 27 bpm wild-type animals control versus cariporide and 470 ± 25 versus 535 ± 20 bpm β_1 -receptor transgenic mice control versus cariporide), $\text{dp}/\text{dt}_{\text{max}}$, or $\text{dp}/\text{dt}_{\text{min}}$ in wild-type animals (Figures 4A through

4C). Thus, hemodynamic unloading appeared unlikely as a major factor contributing to the effect of cariporide.

Discussion

Chronic stimulation of cardiac β_1 -adrenergic receptors plays a crucial part in the development of heart failure. With the present study, we provide evidence that the cardiac $\text{Na}^+\text{-H}^+$ exchanger is involved in the detrimental effects of chronic β -adrenergic stimulation. The main results of this study are that (1) NHE1 expression was upregulated in β_1 -receptor transgenic mice and that (2) treatment with the NHE1 inhibitor cariporide greatly reduced the detrimental effects of chronic stimulation of the β_1 -adrenergic receptor system

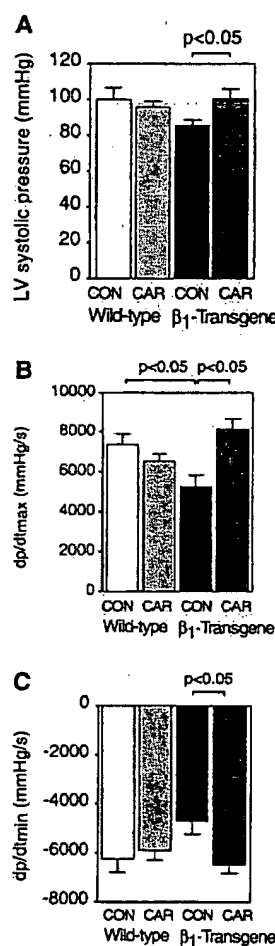


Figure 4. Cariporide prevents the loss of contractile function in β_1 -receptor transgenic mice. Eight-month-old mice were anesthetized with tribromoethanol, and a Millar microtip catheter was advanced through the right carotid artery into the left ventricle. A, Treatment with cariporide prevented the decrease in left ventricular (LV) systolic pressure in β_1 -adrenergic receptor transgenic mice but did not affect LV systolic pressure in wild-type mice. B, β_1 -Adrenergic receptor transgenic mice have reduced LV contractility compared with wild-type mice. Treatment with cariporide prevented this loss of LV function. C, Inhibition of $\text{Na}^+\text{-H}^+$ exchange by cariporide also led to a restoration of diastolic function in β_1 -adrenergic receptor transgenic mice. Data are mean \pm SEM; $n=5$.

including hypertrophy, fibrosis, and the development of heart failure.

The cardiac $\text{Na}^+\text{-H}^+$ exchanger represents one of the heart's key components to maintain physiological intracellular pH. Under conditions of myocardial ischemia, this physiological mechanism seems to exert detrimental effects on the myocardium, probably by increasing intracellular sodium load, which finally results in elevated intracellular calcium via the $\text{Na}^+\text{-Ca}^{2+}$ exchanger.^{8,16} Numerous studies using various NHE1 inhibitors demonstrated protective effects of NHE1 inhibition in animal models of myocardial ischemia (reviewed by Karmazyn et al¹⁶). NHE1 inhibitors have recently also been demonstrated to exert protective actions in a postinfarction model.¹⁴ Although the drug was administered during the ischemic period, there was still a beneficial effect on cardiomyocyte hypertrophy in nonischemic regions of the myocardium. Thus, one might speculate that part of the protective effect of NHE1 inhibition in this model might have been independent from the drug's action on ischemic cardiomyocytes. Indeed, the present work supports the notion that the $\text{Na}^+\text{-H}^+$ exchanger NHE1 is involved in the formation of hypertrophy, fibrosis, and heart failure of nonischemic origin. To our knowledge, this represents the first study that implies the cardiac $\text{Na}^+\text{-H}^+$ exchanger in the detrimental effects of chronic β -adrenergic stimulation. Taking into account the importance of elevated catecholamine levels, and thus adrenergic overstimulation for the progression of human heart failure,⁵ pharmacological inhibition of the cardiac $\text{Na}^+\text{-H}^+$ exchanger might prove useful for the treatment of this disease. Indeed, it has recently been shown that there is increased activity of NHE1 in myocardial samples from patients with human heart failure.¹⁷

Cariporide is a highly selective NHE1 inhibitor with 60-fold selectivity over NHE2 and 3000-fold selectivity over NHE3.^{18,19} With the concentrations used in this study ($2.5 \pm 0.3 \mu\text{mol/L}$ in the plasma), it has to be assumed that NHE1 is the main target of cariporide. The exact mechanism by which NHE1 inhibition exerts its inhibitory effect on β_1 -adrenergic receptor-induced cardiac hypertrophy remains to be determined. Changes in the concentrations of both transported ions, ie, protons and sodium ions, might be involved. Given the high capacity of the other pH-regulating systems in cardiac myocytes under physiological pH, it appears rather unlikely that changes in pH are responsible for the observed alterations. Rather an increased sodium-load could potentially contribute to the detrimental effects observed after chronic β_1 -adrenergic stimulation. Sodium has been shown to exert hypertrophic effects in isolated cardiac myocytes.²⁰ This increased intracellular sodium might be exchanged against calcium via the cardiac $\text{Na}^+\text{-Ca}^{2+}$ exchanger, as has been proposed for the NHE1 activation observed during myocardial ischemia.¹² The resulting increase in diastolic calcium might then exert numerous deleterious effects, including the activation of protein kinase C and calcium-dependent transcription factors. Interestingly, we have found alterations in intracellular calcium handling in β_1 -adrenergic receptor transgenic mice that are similar to human heart failure and include markedly prolonged calcium transients.¹⁵ Furthermore, increased NHE1 activity has been

demonstrated in the myocardium of spontaneously hypertensive rats^{21,22} and after treatment of cardiac myocytes with hypertrophic agonists of Gq-coupled pathways such as endothelin,²³ angiotensin,²⁴ and thrombin.²⁵ Takewaki et al²⁶ showed in cultured myocytes that NHE inhibition partially inhibited stretch-induced activation of MAP-kinases and activation of MAP-kinases was linked to hypertrophic signaling of adrenergic receptors.^{27,28}

The antihypertrophic effect of NHE1 inhibition with cariporide occurred in the absence of any detectable impairment of normal growth of the animal as a whole or of its heart. Thus, the mechanism of action of cariporide must somewhat differentiate between physiological growth (the animals were treated from the age of 3 weeks) and pathological growth of the cardiac myocyte, the latter being caused by chronic β -adrenergic stimulation. How could this be achieved? A potential explanation could reside in our finding that the expression level of NHE1 differs between normal hearts and those undergoing pathological hypertrophy after prolonged β_1 -adrenergic stimulation. Although the level of NHE1 activity is very low under physiological pH,²⁹ the transcriptional activation induced by β -adrenergic stimulation may increase NHE1 activity to a level where it significantly contributes to the development of cardiomyocyte hypertrophy. To our knowledge, this is the first report of β -adrenergic stimulation of NHE1 transcription in the heart. Transcriptional activation of the NHE1 gene has also been shown for a variety of mitogenic and growth-promoting stimuli,³⁰ thus providing a mechanism how cariporide may suppress various forms of cardiomyocyte hypertrophy. The latter is corroborated by our finding that the increased expression of NHE1 was completely normalized after treatment with the NHE1 inhibitor cariporide. This occurred in the presence of continued β -adrenergic signaling, and thus, the expression of the $\text{Na}^+\text{-H}^+$ exchanger might be stimulated by a mechanism further downstream in the signaling cascade.

Another intriguing finding of the present study is the inhibition of interstitial fibrosis by cariporide. Overexpression of the β_1 -adrenergic receptor is targeted to cardiac myocytes in our transgenic model by the use of the murine αMHC promoter. Thus, one possibility is that the observed decrease of interstitial fibrosis under treatment with cariporide reflects a decrease of cardiomyocyte death, which then leads to less replacement fibrosis. However, cariporide might exert additional direct effects on cardiac fibroblasts via a yet undefined mechanism. Cardiac fibroblasts are known to express NHE1³¹, and it has been shown that mitogenic stimuli activate NHE1 in fibroblasts both on the transcriptional and on the protein level.³⁰

We conclude that the $\text{Na}^+\text{-H}^+$ exchanger NHE1 is involved in the hypertrophic and fibrotic structural changes observed after chronic β -adrenergic activation. In addition to its application to prevent postischemic damage to the heart,³² inhibition of NHE activity might represent a novel therapeutic strategy in human heart failure.

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